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SODIUM REQUIREMENTS IN HARDENING OF THE FERTILIZATION ENVELOPE AND EMBRYONIC DEVELOPMENT IN SEA URCHINS

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Abstract

The role of sodium ions in fertilization and development of sea urchin (*Arbacia punctulata* and *Strongylocentrotus purpuratus*) eggs was studied by culturing eggs and embryos in low sodium (choline-substituted) sea water. Hardening of the fertilization envelope was impaired in 19 m*M* Na^+ as indicated by the collapse of this investment 30–60 min after insemination. Cross-linking of the fertilization envelope, assayed in terms of the onset of resistance to dispersal by isotonic urea, was not affected by low sodium. However, impregnation of the fertilization envelope by structural proteins derived from the egg's cortical granules did not take place in low sodium. The "I–T" transition in the configuration of the fertilization envelope in *Strongylocentrotus* from "igloo" shaped casts of microvilli that were at the surface of the unfertilized egg to sharp "tent" shaped spikes also was prevented in low sodium. Potassium and lithium effectively substituted for sodium in promoting the normal structuralization of the fertilization envelope, while choline and Tris did not. Fertilized eggs divided more slowly in low sodium than in normal (419–425 m*M* Na^+) sea water. *Arbacia* embryos fertilized and cultured in low sodium sea water showed a reversible developmental arrest at the swimming blastula stage. This finding suggests that a sodium activated switch initiates gastrulation (differentiation) in sea urchins. Taken together, these results show that sodium is essential for several physiologic processes related to fertilization and developmental in sea urchins, in addition to the previously described rapid-electrical block to polyspermy and the coupling of early and late events in egg activation.

Introduction

Sodium, the most abundant cation in sea water, is known to participate in several processes associated with the development of fertilized sea urchin eggs, including: the sperm-initiated electrical depolarization of the egg's plasma membrane (Chambers and deArmendi, 1979), establishment of a rapid block to polyspermy (Jaffe, 1980; Schuel and Schuel, 1981), and the coupled efflux of protons to link the early and late events of egg activation (Chambers, 1976; Epel, 1978).

A possible role for sodium in hardening of the fertilization envelope was suggested by the observation that this investment tended to crenate and collapse in low sodium (choline-substituted) sea water subsequent to its elevation during fertilization (Nishioka and Cross, 1978). The fertilization envelope is derived in part from the vitelline layer that is attached to the plasma membrane of the unfertilized

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egg as well as secretory products discharged by the egg's cortical granules at fertilization (reviewed by Schuel, 1978). Immediately after elevation the fertilization envelope is soft and easily removed by a variety of physical and chemical treatments, but it gradually hardens and becomes extremely difficult to remove or disperse (Kopac, 1940; Harvey, 1956; Lallier, 1971; Veron *et al.*, 1977; Carroll and Baginski, 1978). These changes are promoted by peroxidatic enzymes (Foerder and Shapiro, 1977; Hall, 1978) and structural proteins (Anderson, 1968; Inoue and Hardy, 1971; Chandler and Heuser, 1980) that are secreted by the egg's cortical granules (Schuel, 1978).

The present communication defines the functional role of sodium ions in hardening of the fertilization envelope, and also identifies sodium requirements for cell division and for gastrulation during subsequent embryonic development in sea urchins. Preliminary accounts of portions of this study have been presented previously (Schuel *et al.*, 1979, 1981).

Materials and Methods

Gametes from the sea urchins *Arbacia punctulata* and *Strongylocentrotus purpuratus* were used in this study. *Arbacia* were collected locally at Woods Hole, MA during the summer months and kept in fresh running sea water. During the winter *Arbacia* (obtained from Florida Bio-specimen Co., Panama City, FL) and *Strongylocentrotus* (obtained from Pacific Bio-Marine Supply Co., Venice, CA) were kept at Buffalo in a marine aquarium (Aquarium Systems, Inc., Wickliffe, OH).

Gametes were obtained by injection of isotonic (0.5 *M*) potassium chloride into the coelomic cavity of adult sea urchins (Harvey, 1956). Semen was collected "dry" and stored over crushed ice. Working sperm suspensions were prepared by diluting the semen with sea water just prior to insemination. The eggs were collected in sea water, filtered through cheese cloth, washed with sea water, and stored over crushed ice until used. The eggs were inseminated and cultured at 24°C for *Arbacia* and 15°C for *Strongylocentrotus*. The culture media consisted of either natural (425 m*M* Na^+) sea water (Cavanaugh, 1964), artificial (419 m*M* Na^+) sea water (Goldstein, 1953), or low sodium (19–26 m*M*) sea water prepared by equimolar substitution of choline chloride for sodium chloride (Chambers and deArmendi, 1979). In certain experiments potassium, lithium, and Tris (tris(hydroxymethyl)aminomethane) were used as sodium substitutes in place of choline (Gould-Somero *et al.*, 1979). Since sea urchin eggs will not fertilize in potassium- or lithium-substituted sea water, they were fertilized in normal artificial sea water and transferred into these solutions at 10 sec after insemination. At this time essentially all the eggs had been activated, while less than 10% of the cortical granules had undergone exocytosis (Dandekar and Schuel, unpublished data). Choline chloride and Tris were obtained from Sigma Chemical Co. (St. Louis, MO).

Hardening (cross-linking) of the fertilization envelope was evaluated on the basis of the onset of resistance to solubilization in isotonic (1.0 *M*) urea (Moore, 1930; Kopac, 1940). The eggs (0.4 ml) were pre-incubated for 5 min in artificial or low sodium sea water (10 ml) and then inseminated by addition of 0.1 ml of 10% sperm. The eggs were removed from the cultures by gentle centrifugation (IEC Clinical Centrifuge, head #221 at setting 5 × 20 sec) at various times post insemination, washed (2 × for 60 sec) in 10 ml of 1.0 *M* urea, returned to sea water, and scored for the presence of the fertilization envelope as observed by light microscopy.

Cleavage time was determined by fixing the cultures with 2% glutaraldehyde at various intervals before, during, and after first cleavage. The eggs were examined by light microscopy and divided zygotes were counted. The time at which 50% of the population had divided was estimated from plots of percent divided *vs* time.

Approximately 100–200 eggs were counted in each culture. The data are presented as mean values plus or minus the standard deviation for the indicated number (N) of experiments. The statistical significance between control and experimental eggs was evaluated on the basis of the "*t*" test for two sample means (Brownlee, 1965).

Ultrastructural observations were performed on eggs fixed and processed for transmission electron microscopy using previously described procedures for *Arbacia* (Longo and Anderson, 1972) and *Strongylocentrotus* (Summers and Hylander, 1974).

Results

The fertilization envelope elevated from *Arbacia* and *Strongylocentrotus* eggs within 60 sec after insemination in both normal (419–425 m*M* Na^+) and sodium-depleted (19–26 m*M*) sea water. In low sodium (choline-substituted) sea water the perivitelline space appeared to be wider, and the fertilization envelope appeared to be thinner and more refractile as observed by light microscopy. The fertilization envelope normally thickens and becomes darker within several min post insemination in normal sea water. However, in low sodium the fertilization envelope remained thin and refractile, and by 15 to 45 min after insemination it began to crenate and collapse onto the surface of the zygote (Fig. 1). Transmission electron microscopy revealed that cortical granule exocytosis was completed by 60 sec after insemination in both normal and sodium-depleted sea water (see Fig. 4A, below). There was no indication that any of the components released by the cortical granules had been precipitated by the choline-substituted sea water. Taken together these observations confirmed the previous findings by Nishioka and Cross (1978) that hardening of the fertilization envelope was impaired in low sodium sea water, and suggested that some process subsequent to the release of the contents of the cortical granules had been affected. Accordingly, we studied the effects of sodium-depleted sea water on two later manifestations of fertilization envelope hardening in sea urchins: structuralization by released cortical granule contents (Anderson, 1968; Inoue and Hardy, 1971), and the onset of reduced solubility in isotonic urea (Kopac, 1940). The latter is a measure of the extent of intermolecular cross-linking (Lorand, 1972).

Transmission electron microscopic observations showed that the gradual structuralization of the fertilization envelope did not take place in choline-substituted low sodium sea water (Figs. 2 and 3). A thin and fluffy fertilization envelope was present in *Arbacia* (compare Figs. 2A and 2B) and *Strongylocentrotus* (compare Figs. 3A and 3B) eggs at 60 sec post insemination in both normal and low sodium sea water. One hour later a thickened tri-laminar fertilization envelope, typical of this matured and fully hardened external investment (Anderson, 1968; Inoue and Hardy, 1971), had developed in normal (419–425 m*M* Na^+) sea water (Figs. 2C and 3C). However, in low sodium (19–26 m*M*) the morphology of the fertilization envelope at one hour had not changed from that observed at 60 sec (compare Figs. 2B and 3B with 2D and 3D). The fertilization envelope in *Strongylocentrotus* (Figs. 3A and 3B), but not in *Arbacia* (Figs. 2A and 2B), initially retains rounded "igloo or I" shaped casts of the microvilli that were at the surface of the unfertilized egg. These casts in *Strongylocentrotus* gradually become "tent or T" shaped as the

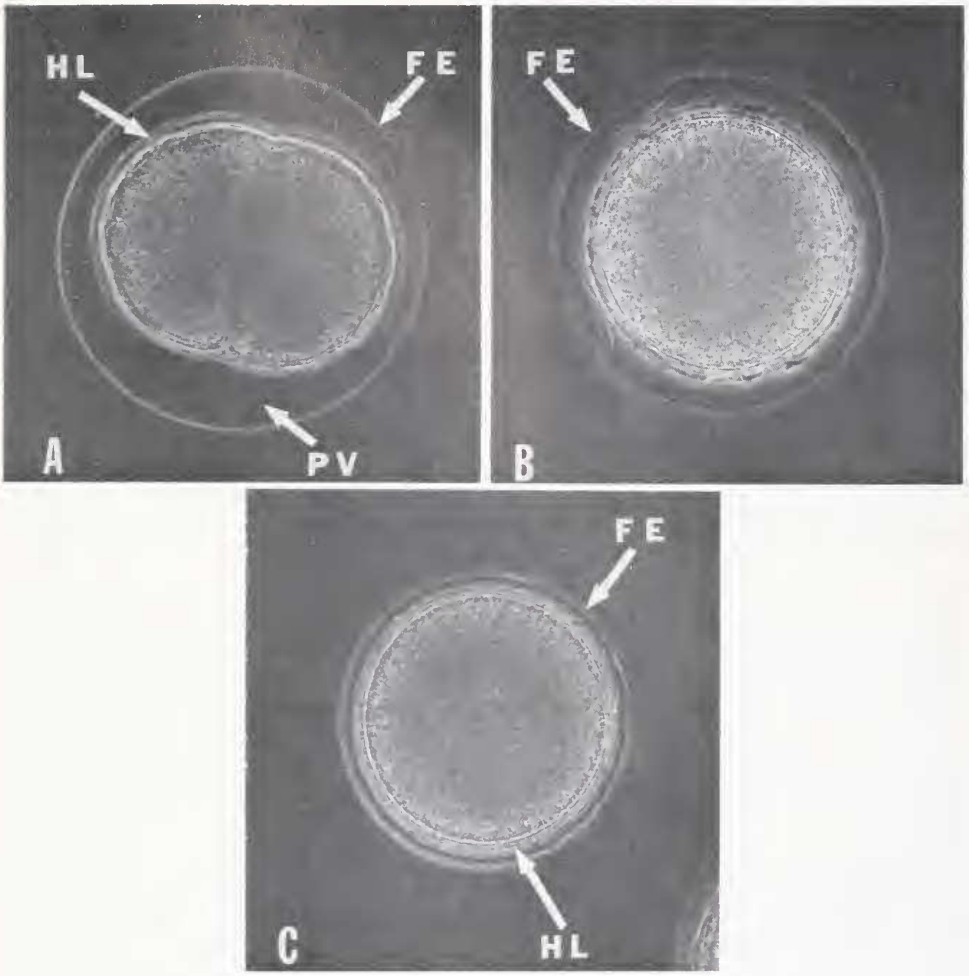


FIGURE 1. Collapse of fertilization envelope in *Strongylocentrotus purpuratus* eggs induced by sodium-depleted (choline-substituted) sea water. Phase contrast micrographs of living eggs photographed at time of first cleavage in 412 m*M* Na^+ sea water (A). Eggs cultured in 19 m*M* Na^+ sea water (B, C) have not begun to divide at this time. Mag. 440 ×.

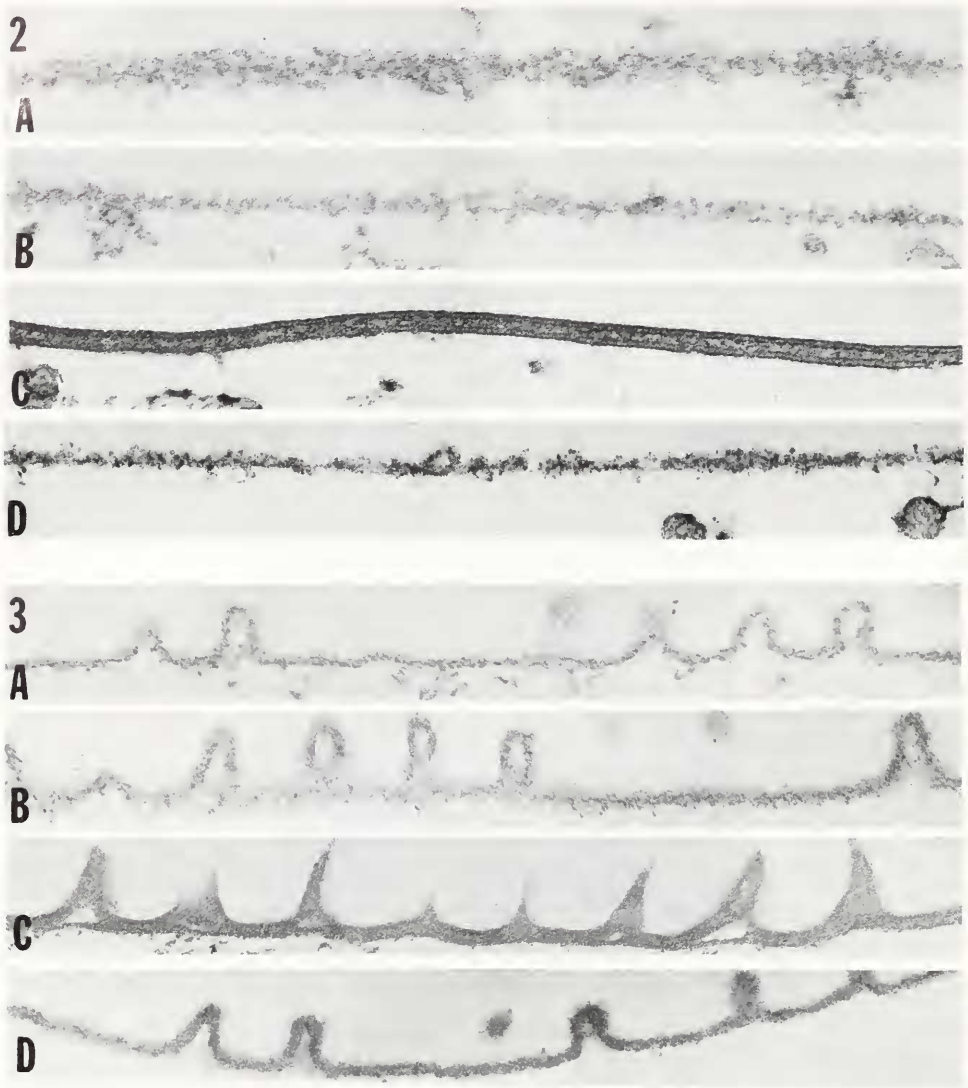
(A): Note the spherical shape of the normally hardened fertilization envelope (FE), fully developed perivitelline space (PV), and the hyaline layer (HL) that is applied to the surface of the dividing zygote cultured in normal sea water.

(B): The fertilization envelope is crenated and partially collapsed in this zygote cultured in low sodium sea water.

(C): The fertilization envelope has completely collapsed onto the hyaline layer in this low sodium sea water treated zygote.

fertilization envelope hardens and structuralizes (Veron *et al.*, 1977). This "I–T" transition in *Strongylocentrotus* does not take place in low sodium (choline-substituted) sea water (compare Figs. 3C and 3D). Similar effects were observed with Tris-substituted low sodium sea water (data not shown). However, when potassium or lithium were used to replace sodium instead of choline the fertilization envelope structuralized normally and did not collapse (data not shown).

Cross-linking of the fertilization envelope, assayed by the loss in urea solubility,



FIGURES 2 and 3. Transmission electron micrographs showing the effects of low sodium (choline-substituted) sea water on structuralization of the fertilization envelope in *Arbacia* (Fig. 2) and *Strongylocentrotus* (Fig. 3). Panels A: normal sea water at 60 sec post insemination: Panels B: low sodium sea water at 60 sec; Panels C: normal sea water at 60 min; Panels D: low sodium sea water at 60 min post insemination. In all micrographs the perivitelline space is at the bottom. Mag. Fig. 2, 64,000 ×; Fig. 3, 40,000 ×.

was not affected by low sodium sea water (Table I). The data show that all the fertilization envelopes were solubilized by 1.0 *M* urea at 1 min after insemination, about half were soluble at 5 min, and all were insoluble by 10 min in both artificial (419 m*M* Na^+) and 19 m*M* Na^+ (choline-substituted) sea water. The removal of the soft fertilization envelope by urea was confirmed by transmission electron microscopy (Fig. 4). Both the fertilization envelope and hyaline layers were completely removed from eggs inseminated in 19 m*M* Na^+ and treated with urea at 60 sec

TABLE I

Effect on sodium-depleted (choline-substituted) sea water on hardening of fertilization envelope in S. purpuratus.

Treatment	Fertilized (% FE)	Urea insoluble fertilization envelopes (%) at min post insemination		
		1	5	10
Sea water (412 m*M* Na^+)	100.0 ± 0.0	0.0 ± 0.0	42.0 ± 16.0	98.9 ± 1.5
Sodium-depleted SW (19 m*M* Na^+)	100.0 ± 0.0	0.0 ± 0.0	59.3 ± 24.0	98.0 ± 2.8
			($P < 0.2$)	($P > 0.5$)

Experimental conditions described in text under Methods.
N = 4.

(Figs. 4A and 4B). When eggs were treated with urea at 10 min after insemination both the fertilization envelope and hyaline layers remained intact (Figs. 4C and 4D). Similar results were obtained with eggs fertilized in 419 m*M* Na^+ (data not shown). There was no morphological difference in the development of the hyaline layers in eggs fertilized in normal and sodium-depleted sea water. The hardening of the hyaline layer is known to be promoted by the formation of cross-links via calcium ions (Citkowitz, 1971).

Low sodium (choline-substituted) sea water also was observed to retard cleavage (see Fig. 1, above) and prevent normal embryonic development. Previous studies showed that sea urchin eggs are extremely vulnerable to polyspermy in sodium-depleted sea water (Jaffe, 1980; Schuel and Schuel, 1981). Furthermore, polyspermic eggs are known to divide more slowly than monospermic zygotes (Schuel *et al.*, 1973; Longo *et al.*, 1974) and rarely develop beyond the blastula stage (Morgan, 1927). Hence, these experiments were conducted with eggs that had been inseminated with a minimal sperm density just sufficient to fertilize all the eggs in low sodium without inducing significant polyspermy.

The retardation of first cleavage in eggs fertilized and cultured in sodium-depleted sea water was quantitatively documented in *Arbacia* (Fig. 5). In three similar experiments the time for 50% of the eggs to divide in natural (425 m*M* Na^+) sea water was 48.2 ± 2.8 min, compared to 62.0 ± 1.3 min in 26 m*M* Na^+ at 24.3 ± 0.6°C ($P < 0.02$). The time required for first cleavage of control eggs in this study is consistent with previous observations on *Arbacia* under similar conditions (Harvey, 1956). The delay in first cleavage induced by sodium-depleted sea water in these experiments was 13.8 ± 3.3 min.

Early development of *Arbacia* zygotes continued in low sodium (26 m*M*) sea water, but was arrested at the swimming blastula stage. This effect was completely reversible and development into mature larvae (plutei) ensued if the embryos were returned to natural sea water anytime between 30 sec to 24 hours after insemination. By 24 hours after insemination in either natural or artificial (425 m*M* Na^+) sea water, gastrulation had been completed and young plutei were actively swimming in the cultures. If 24 hours old *Arbacia* embryos arrested at the swimming blastula stage in 26 m*M* Na^+ were returned to natural or artificial (425 m*M* Na^+) sea water at this time, they resumed development and reached the pluteus larva stage within another 24 hours.

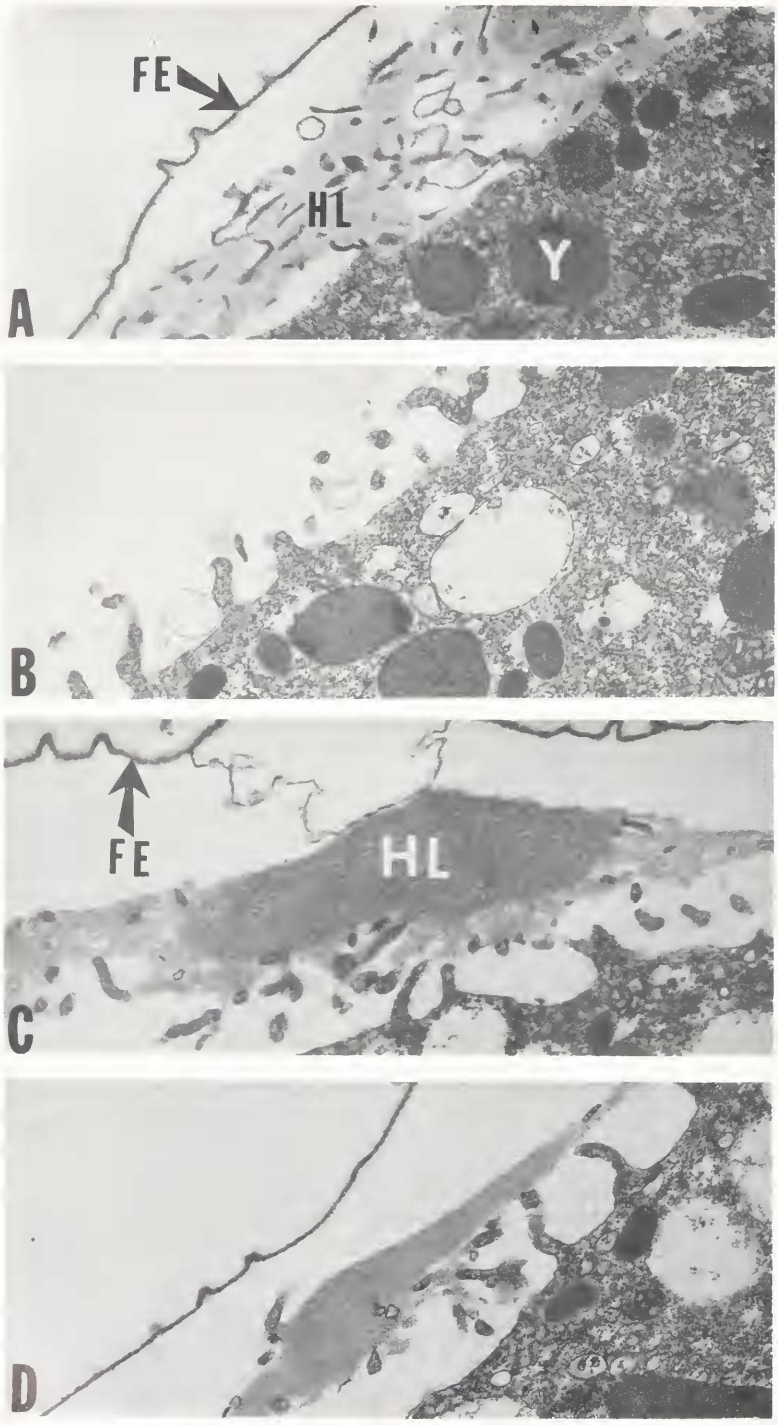


FIGURE 4. Transmission electron micrographs of *S. purpuratus* eggs fertilized in 19 m*M* Na^+ (choline-substituted) sea water and treated with 1.0 *M* urea to remove the fertilization envelope as described in Table I. Mag. 18,000 ×.

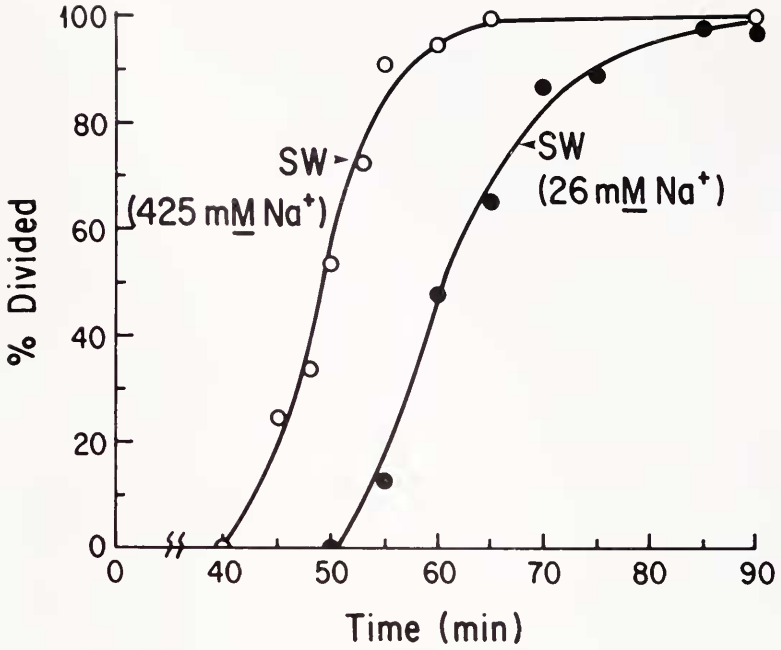


FIGURE 5. Delay in first cleavage of *Arbacia* eggs induced by sodium-depleted sea water. Eggs inseminated with minimal sperm, cultured at 24°C, and fixed at indicated times post insemination.

DISCUSSION

The importance of the common cations in sea water in regulating processes associated with fertilization and embryogenesis in sea urchins has been recognized for many years (see reviews by: Herbst, 1904; Loeb, 1913; Lillie, 1919; Heilbrunn, 1956; Runnstrom, 1966; Epel, 1978; Schuel, 1978). The results of the present study identify additional roles for sodium ions in these phenomena.

Elevation of the vitelline layer from the sea urchin egg surface and its gradual transformation into the fully hardened fertilization envelope is a complex process that is mediated by the secreted products of the cortical granules plus cations in the ambient sea water. Detachment of the vitelline layer from the egg's plasma membrane is promoted by a cortical granule derived serine protease (Longo and Schuel, 1973; Schuel *et al.*, 1973; Carroll, 1976) which requires calcium to maintain its enzymatic activity (Vacquier, 1975). Elevation of the detached vitelline layer is promoted by the secretion of acidic polyanions and other cortical granule derived macromolecules into the perivitelline space (Schuel *et al.*, 1974; Schuel, 1978; Green and Summers, 1980). The subsequent hardening of the fertilization envelope is promoted by other released cortical granule products: a peroxidase which cat-

(A): Egg fixed at 60 sec after insemination. Fertilization envelope (FE), hyaline layer (HL), yolk platelet (Y). Cortical granules have all undergone exocytosis by this time. Degranulation completed by 60 sec in normal (419 m*M* Na^+) sea water (data not shown).

(B): Egg treated with 1.0 *M* urea at 60 sec after insemination. Note the complete removal of fertilization envelope and hyaline layer.

(C): Egg fixed at 10 min after insemination.

(D): Egg treated with 1.0 *M* urea at 10 min after insemination. The fertilization envelope and hyaline layers are intact.

alyzes the formation of dityrosine cross-links (Foerder and Shapiro, 1977; Hall, 1978), and structural proteins that are inserted into the fertilization envelope (Anderson, 1968; Inoue and Hardy, 1971; Chandler and Heuser, 1980). The results of the present study indicate these are separate events in the hardening process. Structuralization appears to be sodium dependent, while cross-linking is not.

The increased resistance of the fertilization envelope to dissolution by urea, as well as other organic dispersing solvents (Lallier, 1971; Foerder and Shapiro, 1977; Carroll and Baginski, 1978; Hall, 1978), during hardening is analogous to that associated with the polymerization of fibrin during the coagulation of mammalian blood (Lorand, 1972). In both systems the resistance to solubilization by 1.0 *M* urea reflects the formation of new covalent bonds to cross-link a previously soft and easily dispersed structure. The results of the present study are consistent with this hypothesis since the urea treatment completely removed the fertilization envelope at 1 min post insemination but was ineffective if applied at 10 min. Our data also show that this transition is not impaired in low sodium (19 m*M*) sea water.

The fertilization envelope normally is impregnated by cortical granule derived structural proteins to form a thickened tri-laminar investment (Anderson, 1968; Inoue and Hardy, 1971; Chandler and Heuser, 1980). Polymerization of the paracrystalline protein that contributes to the structuralization of the fertilization envelope depends upon the divalent cations, calcium and magnesium, found in sea water (Bryan, 1970). Isolated soft (non-cross-linked) fertilization envelopes undergo a "wraithing" process, altered refractility and loss in rigidity, in low sodium media (Kay *et al.*, 1980) which mimics those observed by us *in vivo*. Our results suggest that the actual insertion of cortical granule derived structural proteins into the fertilization envelope as well as the "I–T" transformation are sodium-dependent processes. Potassium and lithium can effectively replace sodium in promoting these structural changes, while choline and Tris can not. Alternatively it may be the presence of choline or Tris rather than absence of sodium that is responsible for the observed defects in assembly of the fertilization envelope. In either case choline- and Tris-substituted sea water should be a useful tool to probe the mechanism for insertion of the cortical granule derived structural proteins into the fertilization envelope during hardening.

The "I–T" transformation of the *Strongylocentrotus* fertilization envelope appears to depend upon the inserted structural proteins. This notion is supported by morphological evidence that these processes take place concurrently during fertilization envelope hardening (Chandler and Heuser, 1980), and by the observation that removal of these structural proteins from the hardened fertilization envelope *in vitro* by repeated extraction with 6.0 *M* urea-1.5 *M* mercaptoethanol (pH 10) at 100°C results in the disappearance of the "T" projections (Carroll and Baginski, 1978). The fertilization envelope in *Arbacia* does not retain casts of microvilli that were at the surface of unfertilized egg and thus does not undergo an "I–T" transformation, but it does structuralize during hardening. This implies a species difference in the properties of the vitelline layer and fertilization envelope between *Arbacia* and *Strongylocentrotus*. However, the rigidity of the hardened fertilization envelope appears to depend upon the inserted structural proteins in both species. Failure of this process in low sodium sea water may account, in part, for the observed crenation and collapse of the fertilization envelope.

Subsequent to these changes, the permeability of the fertilization envelope to proteins gradually is restricted (Veron *et al.*, 1977). This alteration in the fertilization envelope probably prevents the escape of macromolecules from the peri-

vitelline space, and also may act to further insulate the developing embryo from potential hazards in its environment. The hydration of colloidal polyanions that are secreted by the egg into the developing perivitelline space during fertilization is believed to play a major role in the initial elevation of the fertilization envelope (Schuel, 1978). If the restricted permeability of the fertilization envelope does not develop normally in low sodium sea water, then the gradual loss of colloidal polyanions from the perivitelline space could contribute to the collapse of the fertilization envelope.

The cleavage delay and the reversible failure to gastrulate in low sodium (26 m*M*) sea water are processes distinct from the previously documented requirement for a minimum of 3 m*M* Na^+ for the coupled efflux of protons during activation of the egg at fertilization in order to initiate cleavage and development (Chambers, 1976; Epel, 1978). If eggs are transferred to zero sodium sea water at 30 sec after insemination, development of the incorporated male pronuclei, sperm aster formation, fusion of male and female pronuclei, and cell division are suppressed (Carron and Longo, 1980). It is unlikely that such a defect in pronuclear development is responsible for the delay in the first cell division observed in the present study, since fusion of male and female pronuclei takes place at the same time in 19 m*M* Na^+ as in eggs inseminated and cultured in normal sea water (Schuel and Schuel, 1981). Additional work is required to identify the lesion responsible for the delay in cell division promoted by low sodium (19–26 m*M*) sea water.

Developmental arrest of sea urchins at the swimming blastula stage in sodium-depleted sea water (composed of magnesium, potassium, and calcium salts) was first reported by Herbst (1904). More recently Chambers (1976) observed that eggs cultured in choline-substituted low sodium sea water developed into blastulae, but he did not look for effects on later stages of development (Chambers, personal communication). The results of the present study confirm and extend these previous observations by showing that development will resume when arrested blastulae are returned to sea water that contains normal levels of sodium. Other kinds of treatments which produce developmental arrest at the swimming blastula stage in sea urchins prevent the activation of genes which direct the synthesis of new proteins required for differentiation (Gross, 1967). Sodium ions have been reported to selectively activate genes and induce chromosomal puffing in other systems (Lezzi, 1970). Taken together these findings suggest that a sodium-activated switch may regulate similar processes essential for gastrulation in sea urchins. Alternatively, the sodium switch may operate at the translation or post-translation levels. Additional work is required to answer these questions.

Acknowledgments

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